Plant Physiol. (1986) 80, 539–543
0032-0889/86/80/0539/05/$01.00/0

Ethylene-Promoted Conversion of 1-Aminocyclopropane-1-Carboxylic Acid to Ethylene in Peel of Apple at Various Stages of Fruit Development [1]

Received for publication June 3, 1985 and in revised form October 24, 1985

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ABSTRACT

Internal ethylene concentration, ability to convert 1-amino-cyclopropane-1-carboxylic acid (ACC) to ethylene (ethylene-forming enzyme [EFE] activity) and ACC content in the peel of apples (*Malus domestica* Borkh., cv Golden Delicious) increased only slightly during fruit maturation on the tree. Treatment of immature apples with 100 microliters ethylene per liter for 24 hours increased EFE activity in the peel tissue, but did not induce an increase in ethylene production. This ability of apple peel tissue to respond to ethylene with elevated EFE activity increased exponentially during maturation on the tree. After harvest of mature preclimacteric apples previously treated with aminoethoxyvinylglycine, 0.05 microliter per liter ethylene did not immediately cause a rapid increase of development in EFE activity in peel tissue. However, 0.5 microliter per liter ethylene and higher concentrations did. The ethylene concentration for half-maximal promotion of EFE development was estimated to be approximately 0.9 microliter per liter. CO_2 partially inhibited the rapid increase of ethylene-promoted development of EFE activity. It is suggested that ethylene-promoted CO_2 production is involved in the regulation of autocatalytic ethylene production in apples.

In some climacteric fruits, including the apple, sufficient ethylene is present some time before ripening to trigger autocatalytic ethylene production and ripening (6). However, low responsiveness of the fruit tissue to ethylene prevents the onset of autocatalytic ethylene production and ripening. It is thought that apples will remain in an unresponsive state to either endogenous or exogenous ethylene until a hypothetical ripening inhibitor has decreased or some effector has accumulated in the tissue (9, 23). Exogenous or endogenous ethylene, however, is effective in shortening the time to the eventual onset of autocatalytic ethylene production, *e.g.* by destroying the ripening inhibitor (9, 21, 23). Once ripening is induced, ethylene will promote the rate of the various ripening processes including its own biosynthesis (3, 18). This sequence of ethylene action, initiation of ripening followed by promotion of ripening, fits the postulation of McMurchie *et al.* (19) that two systems are involved in the regulation of ethylene biosynthesis in climacteric fruits. System 1 ethylene is related to the low preclimacteric ethylene production and is initiated or controlled by an unknown 'aging' factor. System 1 ethylene then triggers ripening and the formation of vast amounts of system 2 ethylene.

[1] Supported by Deutsche Forschungsgemeinschaft.

The establishment of the pathway of ethylene biosynthesis methionine → SAM[2] → ACC → ethylene (1, 16) led to various studies on the regulation of ethylene biosynthesis in fruits as reviewed by Yang and Hoffman (26). Thus, intact preclimacteric fruits have little ability to convert SAM to ACC or ACC to ethylene and contain only low amounts of ACC. The onset of autocatalytic ethylene production is accompanied by an increase in ACC content as well as an increase in ACC synthase activity and increased conversion of ACC to ethylene (26). Ethylene treatment of preclimacteric cantaloupe or tomato fruit for a short period promotes the ability of the tissue to convert ACC to ethylene without promoting ACC synthesis (14). Ethylene or propylene treatment of apples promotes the development of ACC synthase activity (4, 5).

The present study reports on the effects of ethylene on conversion of ACC to ethylene in intact apples during their development from early preclimacteric (immature) to climacteric (ripening) stage. It has been demonstrated that the ability to convert ACC to ethylene during ripening of intact apples develops similarly in both peel tissue and in cortex tissue (17). This development is closely paralleled by a change in the internal ethylene concentration of the fruit (17). In this respect, since there is no difference between peel tissue and cortex tissue, peel tissue was utilized in this investigation, due to its simplicity in preparation. The ability to convert ACC to ethylene will be denoted in this study as EFE activity (10). Although EFE has not been demonstrated in a cell-free system, its activity can be estimated *in vivo* (10). In the present study some experiments have been performed where preclimacteric apples were treated with AVG to prevent ripening and autocatalytic surge of ethylene production (2). AVG inhibits ethylene biosynthesis by inhibiting the formation of ACC, but does not affect the conversion of ACC to ethylene (1, 10).

MATERIALS AND METHODS

Plant Material. Apple fruits (*Malus domestica* Borkh., cv Golden Delicious) which were similar in size and appearance were selected for the experiments. Apples not treated with AVG were subjected to the indicated assays (at 20°C) within 1 h from picking. The AVG (500 mg active ingredient per L) was sprayed 4 weeks and 2 weeks before harvest on the fruits and leaves.

Chemicals. The AVG, in the form of a wettable powder with 20% active ingredient, was obtained from Dr. R. Maag AG,

[2] Abbreviations: SAM, *S*-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; EFE, ethylene-forming enzyme; AVG, aminoethoxyvinylglycine (2-amino-4-[2-aminoethoxy]-*trans*-3-butenoic acid).

Dielsdorf (Switzerland). The ACC was purchased from Calbiochem and Mes from Serva.

Gas Treatment in a Static System. Six apples per treatment were kept for 24 h in a 21-L desiccator which contained ethylene at a concentration of 100 μl L^{-1} or air. Ethylene was injected immediately after enclosure. In both the air and ethylene treatments, a solution of KOH in a beaker was included to prevent accumulation of CO_2. A solution of $Hg(ClO_4)_2$ was present in the air treatment to prevent accumulation of ethylene. CO_2 concentration in the air and ethylene treatment remained below 0.5 % (v/v). Ethylene concentration in the air treatment remained below 0.05 μl L^{-1}. Ethylene and CO_2 were assayed using a gas chromatograph equipped with a flame ionization detector and a thermoconductivity detector, respectively.

Gas Treatment in a Flow System. Apples were placed in 21-L desiccators and ventilated with a constant flow of moist air (32 L h^{-1}) containing different concentrations of ethylene and CO_2. Ethylene was premixed in cylinders with nitrogen and the desired concentrations were diluted into the air stream by means of a barostat and glass capillaries (22). CO_2 without premixing with N_2, was diluted into the air stream by the same method.

Assay of EFE. Three g of peel strips were cut from the equatorial region of one apple using a domestic potato peeler. The peel strips were then cut with scissors to 3 cm in length and placed with the adhering cortical tissue downward on 0.1 mM ACC dissolved in 10 mM Mes, pH 6.0. After 30 min the peel tissue was quickly blotted with paper towels and enclosed in a 50-ml plastic syringe. Immediately after enclosure, CO_2 was injected into the plastic syringe in order to establish a level of approximately 2% (v/v) CO_2. After 30 min in daylight, the accumulated ethylene in the syringe was determined. Preliminary tests showed no autoinhibitory effect of the ethylene accumulating in the syringe on EFE activity. Under the experimental conditions described, conversions of ACC to ethylene was saturated at approximately 30 mM ACC in the incubation medium. EFE activity (the ability to convert ACC to ethylene) was expressed as nl ethylene g^{-1} h^{-1}. The EFE assay was performed at 20°C.

Determination of Internal Ethylene Concentration of Apples. Internal gas was sampled with a syringe from the core of apples immersed in water. The ethylene concentration of the gas sample was determined by GC.

Determination of Wound-Induced Ethylene Production of Peel Tissue during Aging on Buffer. Peel tissue (3 g) of individual apples was incubated in the dark on 10 mM Mes, pH 6.0, containing 40 μg chloramphenicol per ml. During a 26 h incubation period, ethylene production from the peel tissue was followed by enclosing it at various times for 30 min in a 50-ml plastic syringe and determining the accumulated ethylene.

Extraction and Assay of ACC. Peel strips were cut from the same apples as were used for the EFE assay. Three g were homogenized in 10 ml of 80 % (v/v) methanol using an Ultra Turrax homogenizer. The homogenate was centrifuged and the supernatant evaporated to dryness. The dried residue was dissolved in water and the ACC determined according to Lizada and Yang (15).

With the exception of the ethylene responsiveness experiment (Fig. 3; only 1984) all experiments were conducted in two seasons (1983 and 1984) with similar results. The results presented here are from 1984.

RESULTS

Wound Ethylene Production and EFE Assay. Peel tissue from preclimacteric apples starts to produce significant amounts of wound ethylene approximately 4.5 h after cutting (Table I). At the same time wound-induced development of EFE activity increases rapidly. Therefore, to keep wound-induced development of EFE activity to a minimum, the EFE assay procedure was restricted to a time span of approximately 1 h starting from peeling the fruit. Since peeling of apples can be done rapidly, the use of peel tissue ideally suits this requirement.

Table I. *Wound Ethylene Production and EFE Activity of Apple Peel Tissue*

Peel tissue was incubated on buffer immediately after cutting and its wound ethylene production repeatedly determined by enclosing it for 30 min in a plastic syringe. For estimation of EFE activity peel tissue was continuously incubated on 0.1 mM AVG dissolved in buffer. At various times peel tissue was incubated for 30 min on 0.1 mM AVG plus 0.1 mM ACC and then enclosed for 30 min in a plastic syringe. EFE activity is expressed as the ethylene produced. Ethylene production of peel tissue incubated continuously on 0.1 mM AVG did not exceed 0.2 nl ethylene g^{-1} h^{-1}.

Time after Cutting	Wound Ethylene	EFE Activity
h	*nl ethylene g^{-1} h^{-1}*	*nl ethylene g^{-1} h^{-1}*
0.5	0.21 ± 0.03	
1.0	0.11 ± 0.02	0.25 ± 0.03
3.0	0.32 ± 0.02	0.50 ± 0.05
4.5	0.55 ± 0.06	1.82 ± 0.53
9.0	9.1 ± 2.0	12.3 ± 1.8

Modulation of EFE Activity in Apple Peel Tissue by CO_2. Similar to rice leaf segments and tobacco leaf discs (11), CO_2 seems also to activate ACC conversion to ethylene in apple peel tissue, regardless whether cut from preclimacteric or postclimacteric apples. Apparent saturation is reached at approximately 1.5% (v/v) CO_2 in the surrounding atmosphere for apple peel tissue (data not shown), in contrast to 0.8% (v/v) CO_2 for leaf tissue (11). The explanation for this difference may be found in different parameters of CO_2 diffusion in peel and leaf tissue. Apples readily produce CO_2. This process may be enhanced by ethylene (Fig. 4). Therefore, in this study the CO_2 concentration in EFE assays was generally adjusted to 2% (v/v) CO_2 in order to exclude uncontrolled effects of endogenous CO_2. No significant difference in ACC conversion rate in either light or dark has been found (data not shown).

Ethylene-Forming System in Apples during Their Maturation on the Tree. Apart from occasional fluctuations, the internal ethylene concentration of freshly picked apples revealed only a slight but consistent increase during maturation (Fig. 1A). The ACC content of peel tissue showed a similar pattern (Fig. 1B). EFE activity was constantly low, except for the last sampling date when it almost doubled (Fig. 1A). At this time, 3 out of 10 apples already had an internal ethylene concentration in excess of 0.1 μl L^{-1} and were therefore excluded from analysis. If preclimacteric apples were left to ripen on the laboratory bench at 20°C, individual fruits could be found after a few days with an increased EFE activity (2.5 ± 0.6 nl ethylene g^{-1} h^{-1}), a reduced peel ACC content (0.05 ± 0.01 nmol ACC g^{-1}) and an internal ethylene concentration still below 0.1 μl L^{-1} (compare with Fig. 1). This could suggest an increase of EFE activity before an increase in ACC synthase activity which is accompanied by the autocatalytic surge of ethylene production. Although internal ethylene concentration of apples increased only slightly during maturation (Fig. 1A), the capacity of peel tissue to produce wound ethylene changed significantly during the same time period (Fig. 2). Higher wound-induced ethylene production in mature fruit compared to immature fruit has been reported for various climacteric fruits (10). The increase of wound-ethylene production from peel tissue of immature apples (124 d after full bloom) was slower and had lower peak values compared to peel tissue from apples harvested later (Fig. 2). Thus, ethylene pro-

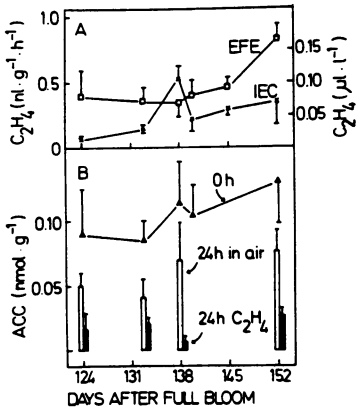

FIG. 1. Internal ethylene concentration (IEC, in $\mu l\ L^{-1}$) of apples, EFE activity (A) and ACC content (B; Oh) of apple peel tissue during maturation. Within 1 h from picking the internal ethylene concentration of the apples was determined and peel tissue of the same apples was cut for EFE assay and ACC extraction. The columns (B) represent ACC contents of peel tissue from apples placed after picking for 24 h in air (open bars) or in 100 $\mu l\ L^{-1}$ ethylene (shaded bars). Each value of internal ethylene concentration and EFE activity represents the mean ± SE of ten apples. Each value of ACC content represents the mean ± SE of 5 apples.

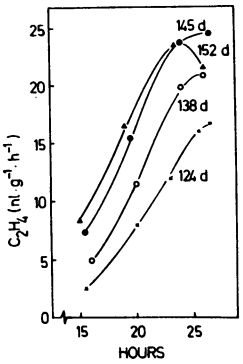

FIG. 2. Wound-induced ethylene production of peel tissue from apples picked at various stages of their maturation (days after full bloom). Peel tissue from individual apples was incubated on buffer and its rate of ethylene production repeatedly determined over a period of 26 h. Each value represents the mean rate of ethylene production of peel tissue from 10 apples.

duction of intact apples is confined to a more or less constant level during maturation, while the capacity for wound-ethylene production increases.

Effect of Ethylene Treatment on EFE in Peel Tissue of Apples during Their Maturation on the Tree. A 24-h treatment of apples with ethylene (100 $\mu l\ L^{-1}$) led to a pronounced increase of EFE activity in peel tissue, depending on the state of maturity of the apples (Fig. 3). The maturity-dependent promotion of EFE activity by ethylene suggests an exponentially increasing response of apples to ethylene estimated as EFE activity. The development of EFE activity is expressed in Figure 3 also as log EFE. Ethylene production of peel tissue was not significantly increased (below 0.1 $nl\ g^{-1}\ h^{-1}$) and ACC content was generally decreased by more than 80% after the 24-h ethylene treatment (Fig. 1B).

Effect of Ethylene on CO_2 Production of AVG-Treated Apples. AVG-treated apples were harvested 146 d after full bloom (October 16, 1984). Their internal ethylene concentration was only 0.011 ± 0.005 $\mu l\ L^{-1}$, whereas that of untreated fruit was 0.060 ± 0.020 $\mu l\ L^{-1}$. Starting 1 d after harvest, AVG-treated apples were ventilated continuously with air or with different concentrations of ethylene in air and their CO_2 production rates were recorded. Except for air and perhaps ethylene at 0.05 $\mu l\ L^{-1}$,

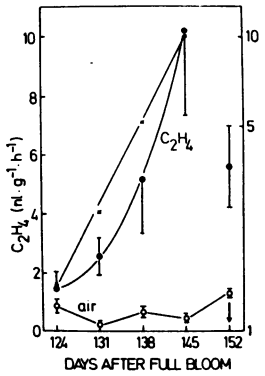

FIG. 3. EFE activity in peel tissue of apples in response to a 24-h ethylene treatment. Apples were picked at various stages of maturity and treated for 24 h with air or 100 $\mu l\ L^{-1}$ ethylene in a closed desiccator. The straight line represents the log transformation of EFE activity after 24 h of ethylene treatment. Each value represents the mean ± SE EFE activity from 7 apples. The arrow indicates onset of increased ethylene production in 30% of the apples at harvest.

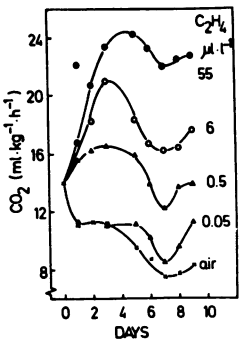

FIG. 4. Effects of different ethylene concentrations on CO_2 production of AVG-treated apples. CO_2 was determined in the air stream leaving the apple-containing desiccators.

each ethylene concentration caused a respiratory increase (Fig. 4). A semilog plot, as adopted by Goeschl and Kays (8), in which increase of respiration is plotted against corresponding logarithm of ethylene concentration, reveals a half-maximal stimulation of CO_2 production at an ethylene concentration of approximately 1.0 $\mu l\ L^{-1}$ and saturation between 15 and 20 $\mu l\ L^{-1}$. Ethylene treatment causes AVG-treated apples to ripen (3).

Effect of Ethylene on Development of EFE Activity. If AVG-treated apples were ventilated continuously with an ethylene concentration of 0.05 $\mu l\ L^{-1}$, EFE activity in peel tissue developed slowly for about 15 d after which a rapid increase to a higher level of EFE activity occurred (Fig. 5). In contrast, higher concentrations of ethylene caused a direct increase in EFE activity to a final level (Fig. 5). It seems, therefore, that ethylene at 0.05 $\mu l\ L^{-1}$ is insufficient to rapidly induce EFE activity in preclimacteric apples as observed with higher ethylene concentrations. A semilog plot (8) shows half-maximal promotion of EFE development by ethylene at approximately 0.9 $\mu l\ L^{-1}$ and saturation close to 20 $\mu l\ L^{-1}$. After repeated evacuation and subsequent shifting of apples from the ethylene (50 $\mu l\ L^{-1}$) treatment into a stream of ethylene-free air (below 0.005 $\mu l\ L^{-1}$), EFE activity in the peel declined by half in approximately 5 d (Fig. 5). Ethylene-promoted EFE activity in preclimacteric cantaloupe or tomato fruit also required the continuous presence of ethylene for maximal response (14).

Effect of CO_2 on Development of Ethylene-Enhanced EFE Activity. CO_2 retards the development of ACC synthase activity in ripening apples and reduces the ethylene-enhanced ACC synthase activity in the presence of ethylene (4). If AVG-treated

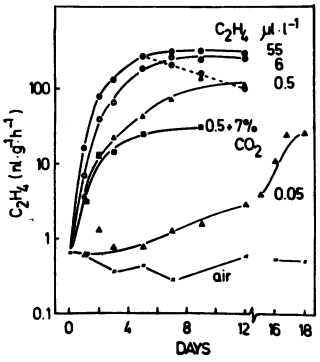

FIG. 5. Effects of different ethylene concentrations and 7% CO_2 on development of EFE activity in peel tissue of AVG-treated apples. The dotted line represents development of EFE activity after transferring apples from ethylene (5 d in 55 $\mu l\ L^{-1}$) into air. Each value represents the mean EFE activity from 5 apples.

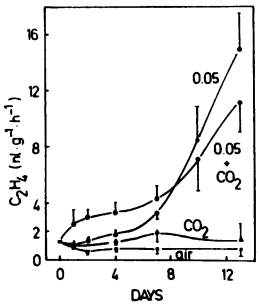

FIG. 6. Effect of ethylene at 0.05 $\mu l\ L^{-1}$ and 7% CO_2 on development of EFE activity in peel tissue from AVG-treated apples. In contrast to other experiments, apples were not used immediately after harvest, but stored for 3 weeks in air at 20°C. Each value represents the mean ± SE EFE activity from 5 apples.

apples were ventilated with ethylene at 0.5 $\mu l\ L^{-1}$ and 7% CO_2, the development of EFE activity was not retarded but the final level of EFE activity was reduced by approximately 70% compared to apples ventilated with ethylene at 0.5 $\mu l\ L^{-1}$ (Fig. 5). Surprisingly, 7% CO_2 in the presence of ethylene at 0.05 $\mu l\ L^{-1}$ had a promoting effect on EFE activity in a first phase and appeared to inhibit in a second phase when the rapid increase of EFE occurred (Fig. 6). It should be noted that the apples used in this experiment (Fig. 6) were stored after harvest for 3 weeks in a stream of moist air until the start of the experiment. This may explain the earlier onset of the rapid rise of ethylene-enhanced EFE activity (after 6 d; Fig. 6), compared to apples treated from 1 d after harvest with ethylene at 0.05 $\mu l\ L^{-1}$ (after 15 d; Fig. 5).

DISCUSSION

The distinction of the ethylene production of climacteric fruits into system 1 and system 2 as proposed by McMurchie *et al.* (19) will serve as a guideline in the discussion of the results.

Preclimacteric ethylene production (system 1) in apples is restricted to a low and almost unchanged level until onset of ripening (fig. 1A). However, the increasing capacity of apple peel tissue during fruit maturation to produce wound ethylene (Fig. 2) and to respond to ethylene treatment with elevated EFE activity (Fig. 3), discloses a gradual release of the ethylene-producing system from restriction. The response of apples to ethylene treatment, estimated by the ability of ethylene to promote development of EFE activity, appears to increase during maturation in an exponential manner (Fig. 3). This may be due to either an increase of the sensitivity or to an increase in the number of ethylene receptors (12, 25). However, the autocatalytic ethylene production does not begin before the fruits' responsiveness to ethylene has reached a certain stage of development (Fig. 3). It is thought that at this stage the endogenous ethylene (system 1) triggers the system 2 ethylene production (18, 19). This stage must have been surpassed by 30% of the fruits 152 d after full bloom (Fig. 3).

However, why then did AVG-treated apples not respond immediately after harvest (146 d after full bloom) to 0.05 $\mu l\ L^{-1}$ ethylene with a rapidly increasing EFE activity (Fig. 5)? A rapidly increasing development of EFE activity is interpreted as indicating induced system 2 ethylene production (see below). Since in natural ripening the endogenous ethylene (system 1) may also increase the responsiveness of the tissue to ethylene (21), it is possible that the responsiveness of AVG-treated apples developed at a slower rate than that of untreated apples. This is because AVG treatment reduced the internal ethylene concentration by 80% (see "Results"). Probably for this reason, AVG-treated apples did not immediately respond to ethylene at 0.05 $\mu l\ L^{-1}$ as would be expected from a fully responsive tissue.

In apples, the onset of autocatalytic ethylene production does not occur before the responsiveness of the tissue to ethylene is fully developed (Fig. 3). This suggests a 'switch-on' mechanism of autocatalytic ethylene production in natural ripening. If increasing responsiveness depends on the increasing amount or sensitivity of an ethylene receptor, then the amount or sensitivity of this receptor must critically change at the onset of autocatalytic ethylene production. This change would signify the switch from system 1 to system 2. Subsequently, development of EFE, ACC synthase and ripening would be set in motion by the ethylene already present in the tissue (system 1 ethylene). The biphasic development of EFE activity as shown in Figures 5 and 6 probably exemplifies such a switch from system 1 to system 2. It appears that this switch is also reflected in the action of CO_2 on ethylene-promoted development of EFE activity. While promotion of EFE seems to be additionally promoted by CO_2 during the first phase (system 1; Fig. 6), promotion of EFE is partially inhibited by CO_2 during the second phase (system 2; Figs. 5 and 6). Although the ethylene-inhibiting action of CO_2 is not fully understood, CO_2 seems either to compete directly with ethylene for its binding site (7) or to act in a more indirect manner (24). The apparent promotion of ethylene action by CO_2 in preclimacteric apple peel tissue requires further study. Promotion of ethylene action by CO_2 has been reported for example in ethylene-stimulated growth rate of rice coleptiles (13) and in overcoming thermodormancy of lettuce seeds (20).

The action of ethylene in system 2 can be further characterized. The half-maximal concentrations of ethylene for the development of ACC synthase and of EFE activities in apples are 0.8 $\mu l\ L^{-1}$ (at 25°C [4]) and 0.9 $\mu l\ L^{-1}$ (from Fig. 5), respectively. In addition, CO_2 production in apples is promoted half-maximally at 1.0 $\mu l\ L^{-1}$ (from Fig. 4). Ethylene-promoted CO_2 production, ACC synthase activity (4), and EFE activity (Fig. 5), all require the continuous presence of ethylene for a maximal response. The striking similarity of the dose-response relationship of these three ethylene effects suggests one ethylene receptor, implying one primary mechanism of ethylene action in system 2 ethylene

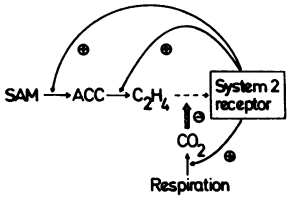

FIG. 7. A simple model accounting for the effects of ethylene and CO_2 in autocatalytic ethylene production (system 2) of apples. ⊕ represents promotion/induction and ⊖ represents inhibition/repression.

production. This view is supported by the effect of CO_2 which counteracts the development of both ethylene-promoted development of ACC synthase activity (4) and of EFE activity (Fig. 5). Whether CO_2 similarly affects ethylene-promoted CO_2 production may be assumed, although experimental evidence is lacking.

However, by inhibition of autocatalytic ethylene production ethylene-promoted CO_2 production could at least be indirectly reduced by CO_2. This situation is depicted in Figure 7. Accordingly, the action of ethylene in autocatalytic ethylene production (system 2) results in promotion of enzyme development required for its own biosynthesis. CO_2 production is concomitantly promoted. This is in agreement with the simultaneous increase of CO_2 production and autocatalytic ethylene production as observed in apples (23). Concentrations of CO_2 in the core of apples range from 2% (v/v) in preclimacteric apples to 10% (v/v) in apples at the climacteric peak, sufficient to hamper ethylene action (7). During ripening ethylene will therefore at the same time promote and reduce the rate of its own biosynthesis (Fig. 7). Moreover, EFE activity seems to be modulated by CO_2 directly (11). This effect of CO_2 is apparently saturated at 1.5% (v/v) or below (see "Results") and will therefore not be limiting during ripening of intact apples. Nevertheless, CO_2 may play a significant role in the ethylene physiology of bulky fruits.

Acknowledgments—I thank Professor Bangerth for broad-minded support and Dr. R. Maag AG for providing AVG.

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